Determination of neutralizing antibodies against IL-6 receptor inhibitor using *iLite*® IL-6 Assay Ready Cells

For research and professional use only. Not for use in diagnostic procedures.

This application note contains a suggested protocol and performance data.
Each individual laboratory must set up their own method and perform relevant validations.

Background

Interleukin-6 (IL-6) is member of the cytokine family. It is found relevant to many inflammatory diseases and many cancers and can act as both a pro-inflammatory cytokine and an anti-inflammatory myokine. In classic signaling, IL-6 stimulates target cells via a membrane bound interleukin-6 receptor (IL-6R), which upon ligand binding associates with the signaling receptor protein gp130. The anti-inflammatory activities of IL-6 are transmitted by this signaling pathway whereas pro-inflammatory responses of IL-6 are mediated by trans-signaling (cells expressing only gp130 respond to a complex of IL-6 bound to a soluble form of the IL-6R). This is important since therapeutic inhibition of IL-6 by the neutralizing anti-IL-6R monoclonal antibody has been focused on the treatment of inflammatory diseases (1).
One of these therapeutic antibodies is tocilizumab, an immunosuppressive drug, consisting of a humanized monoclonal antibody directed against the IL-6R binding. Tocilizumab binds both soluble and membrane bound IL-6R, and inhibit this way the pro-inflammatory effects of IL-6 (2). Prolonged therapies with a biological IL-6R inhibitor may lead to development of neutralizing antibodes (NAbs), which may counteract the IL-6R antagonist activity of the inhibitor. The *iLite*® IL-6 Assay Ready Cells can be used to detect presence of neutralizing antibodies towards IL-6R inhibitors.

Principle of the assay

The *iLite*® IL-6 Assay Ready Cells are engineered cells optimized to express Firefly luciferase under the control of a IL-6 responsive promoter. Binding of IL-6 to the membrane bound IL-6R (IL-6RA and IL6ST) results in activation of the IL-6 regulated Firefly luciferase reporter gene construct. *iLite*® IL-6 Assay Ready Cells also contain the Renilla Luciferase (RL) reporter gene, under the control of a constitutive promoter. The constitutive expression of RL allows normalization of IL-6 induced FL activity, and renders assay results independent of variations in cell number or serum matrix effects. The Firefly luciferase signal can be measured in a luminometer following addition and incubation of luciferase substrate. The Firefly luciferase signal is proportional to the functional activity of IL-6 in the sample. In the presence of inhibitory activity against the IL-6R, the functional activity of the present IL-6 is reduced, resulting in a decreased stimulation of Firefly luciferase production.

The Firefly luciferase signal is inversely proportional to the amount of inhibitory activity in a sample. In the absence of IL-6R inhibitor activity and suspected NAb presence in test samples, a known amount of IL-6R inhibitor is added to quench the Firefly signal and the presence of NAbs is measured as a restored signal. The *iLite*® IL-6 Assay Ready Cells can therefore be utilized as an assay for determination of neutralizing antibodies against IL-6R inhibitors in test samples, including human serum.

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Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® IL-6 Assay Ready Cells	Svar Life Science	BM4061
Diluent (DMEM containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin).	Gibco	31966-021 (DMEM) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Anti-tocilizumab antibody	Bio-rad	HCA253
Tocilizumab	NA	NA
IL-6 or analogues	R&D Systems	206-IL
Firefly substrate	Promega	E2620, Bright-Glo Luciferase Assay System
Pre-incubation plate; PP-microplate, U-shape, 96 well	Greiner bio-one	650261
Plate; White walled micro well plate suitable for luminescence	PerkinElmer	6005680
Microplate Luminometer with appropriate reading software – no filter on luminometer	Contact Svar Life Science for list of recommended suppliers	NA
Incubator, 37 °C with 5% CO_2	NA	NA
Water bath, 37 °C	NA	NA
Single-channel and multi-channel pipettes with polypropylene disposable tips	NA	NA
Polypropylene tubes or plate for dilution	NA	NA
Single-use polypropylene reservoir	NA	NA
Plate shaker	NA	NA
Timer	NA	NA

Protocol

Preparation of neutralizing antibodies against IL-6R inhibitor

An anti-tociluzuzumab antibody from Bio-rad has successfully been used to neutralize tocilizumab (IL-6R inhibitor) and restore the IL-6 regulated Firefly luciferase expression in *iLite*® IL-6 Assay Ready Cells (refer to the table and graph below).

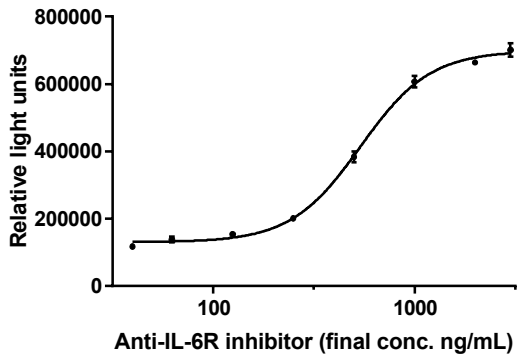

Figure 1. Example of anti-IL-6R inhibitory curve

Final 500 pg/mL IL-6 and 500 ng/mL Tocilizumab	Anti-tocilizumab Suggested solution concentrations, **ng/mL**
A	24 000
B	16 000
C	8 000
D	4 000
E	2 000
F	1 000
G	500
H	0

Table 1. Suggested calibrator **solution** concentrations for anti-tocilizumab

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Incubation

1. Design a plate layout. It is recommended to perform the test at least in duplicate.
2. Perform a serial dilution of the reference anti-tocilizumab antibody. Ensure matrix consistency between reference antibody solutions, control solutions, and sample solutions.
3. Add 20 µL of the reference anti-tocilizumab antibody dilutions, controls and samples to assigned wells on a PP-microplate (final concentration will be one-eighth of solution concentration).
4. Add 20 µL of 4000 ng/mL tocilizumab to all wells (final concentration will be 500 ng/mL tocilizumab).
5. Cover the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2.
6. From each well transfer 20 µL anti-tocilizumab/tocilizumab solution from PP-microplate to white walled microplate.
7. Thaw the vial of *iLite*® IL-6 Assay Ready Cells in a 37°C water bath with gentle agitation. The cell suspension is mixed very carefully ten times with pipette in order to ensure a homogeneous distribution of cells.
8. Dilute 250 µL cell suspension with 2.75 mL Diluent.
9. Add 20 µL diluted cells to each well of the white walled microplate.
10. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2.
11. Add 40 µL of 1000 pg/ml IL-6 to all wells (final concentration will be 500 pg/mL IL-6).
12. Place the lid on the plate, mix and incubate for 6 hours at 37 °C with 5% CO_2.

Adding substrate solutions

1. Equilibrate the plate and the substrate solution to room temperature.
2. Prepare the **Firefly luciferase** substrate according to the suppliers instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.
3. If appropriate, prepare the **Renilla luciferase** substrate according to the suppliers instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.

Precautions

- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according to the supplier's/manufacturer's instructions or product specifications accompanying the individual material and instruments.
- Dispose of all sample specimens, infected or potentially infected material in accordance with good microbiological practice. All such materials should be handled and disposed as though potentially infectious.
- Residues of chemicals and preparations are generally considered as biohazardous waste and should be inactivated prior to disposal by autoclaving or using bleach. All such materials should be disposed of in accordance with established safety procedures.

Proprietary Information

In accepting delivery of *iLite*® Assay Ready Cells the recipient agrees not to sub-culture these cells, attempt to sub-culture them or to give them to a third-party recipient, and only to use them directly in assays. *iLite*® cell-based products are covered by patents which are the property of Svar Life Science AB and any attempt to reproduce the delivered *iLite*® Assay Ready Cells is an infringement of these patents

QUICK GUIDE

Determination of neutralizing antibodies against IL-6R inhibitor using *iLite*® IL-6 Assay Ready Cells

1 Plate 1

- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage.**
- Serial dilute reference anti-tocilizumab antibody.
- Add 20 µL of ref. anti-tocilizumab antibody solutions, controls and samples to pre-assigned wells.
- Add 20 µL of tocilizumab to each well.

2 Incubation 1 30 min

- Incubate at 37 °C with 5% CO_2 for 30 minutes.

3 Plate 2 Add cells

- Transfer references, controls and samples to new wells.
- Thaw the vial of cells in a 37°C water bath. Mix cell suspension with a pipette in order to ensure a uniform cell suspension. Dilute the cells.
- Add 20 µL diluted cells to each well.

4 Incubation 2 30 min

- Incubate at 37 °C with 5% CO_2 for 30 minutes.

5 Add IL-6 Incubation 3 6 hours

- Add 40 µL IL-6 to each well.
- Incubate at 37°C with 5% CO2 for 6 hours.

6 Read plate

- Equilibrate the plate to room temperature
- Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix. Protect the plate from light. After 10 min incubation read in a luminometer.
- If appropriate, prepare the **Renilla luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix. Protect the plate from light. After 10 min incubation read in a luminometer

Troubleshooting and FAQ

Please consult the Svar Life Science website www.svarlifescience.com

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References

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2. Jones, G; Sebba, A; Gu, J; Lowenstein, MB; Calvo, A; Gomez-Reino, JJ; Siri, DA; Tomsic, M; et al, "*Comparison of tocilizumab monotherapy versus methotrexate monotherapy in patients with moderate to severe rheumatoid arthritis: The AMBITION study*". Annals of the Rheumatic Diseases. 69 (1): 88–96. (2010)